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Original article

Influence of bestatin, an inhibitor of aminopeptidases, on T and B lymphocyte subsets in mice

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Abstract

Bestatin, a low-molecular weight dipeptide, is a potent inhibitor of aminopeptidase N which has been demonstrated to have antitumor and immunomodulatory effects. The effects of bestatin (10, 1 and 0.1mg/kg) administered intraperitoneally once, five or ten times to mice on the total number of lymphocytes in the thymus, spleen and mesenteric lymph nodes and the percentage and the absolute number of T cell subsets ($CD4^+CD8^+$, $CD4^-CD8^-$, $CD4^+$, $CD8^+$) in the thymus and T ($CD3^+$, $CD4^+$, $CD8^+$) and B ($CD19^+$) lymphocytes in the spleen and mesenteric lymph nodes were studied. It has been found that bestatin administered ten times at doses of 10, 1 and 0.1 mg/kg increased the total number of thymocytes, splenocytes and lymphocytes of mesenteric lymph nodes. Bestatin also changed the percentage and the absolute number of T cell subsets in the thymus and T and B lymphocytes in the peripheral lymphatic organs. Five and ten exposures to bestatin (10, 1 and 0.1 mg/kg) increased the absolute count of both immature $CD4^+CD8^+$ and $CD4^-CD8^-$ thymic cells. Moreover, both a single and multiple administration of bestatin (1 and 0.1 mg/kg) decreased the percentage and absolute count of $CD3^+$ splenocytes and mesenteric lymph node cells with corresponding decreases in the percentage and absolute count of $CD4^+$ and $CD8^+$ cells. Both a single and multiple administration of bestatin at all the doses under investigation augmented the percentage and the absolute count of $CD19^+$ (B lymphocytes) in the peripheral lymphatic organs. The results of the study show that there is a relationship between the effect induced by bestatin and the dose of the drug as well as the number of doses applied. The strongest effect on the T and B lymphocyte subsets was noted after five injections of bestatin at doses of 1 and 0.1 mg/kg.

Key words: bestatin, B and T lymphocyte subsets, mice

Introduction

The pharmacological modulation of the immune system by natural as well as synthetic agents may be potentially important for the management of certain illnesses, e.g. acquired immune deficiency, infections, allergy, autoimmunological and neoplastic diseases.

Bestatin (ubenimex) is one of the substances believed to be a biological response modifier (BRM). It is a dipeptide N-[(*2S, 3R*)-3-amino-2-hydroxy--4-phenylbutyryl]-L-leucine which has been demonstrated to have antitumor and immunomodulatory effects (Suda et al. 1976). It was isolated from a culture filtrate of *Streptomyces olivoreticuli* (Umezawa et al.

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1976). The immunomodulatory action of bestatin has been confirmed in many studies. Ishizuka *et al.* (1980a) reported that bestatin administered orally at doses of 1, 10 and 100 μg/mouse enhanced delayed-type hypersensitivity (DTH) to sheep red blood cells (SRBC) and was able to restore impaired DTH to SRBC and delayed cutaneous hypersensitivity (DCH) to oxazolone. *In vitro* investigations have shown that bestatin at a concentration of 50 μg/ml inhibited the production of IL-6, IL-8 and MIP-1α (macrophage inflammatory protein) by LPS-stimulated human monocytes and alveolar macrophages from patients with sarcoidosis, while the synthesis of IL-10 by activated monocytes is stimulated (Lkhagvaa et al. 2008). The drug enhances the migration (spontaneous and in the presence of chemotactic stimuli) and phagocytosis of human granulocytes (Jarstrand and Blomgren 1981), exerts a mitogenic effect on lymphocytes (Ishizuka et al 1980b, Weissmann et al. 1985) and augments hematopoiesis (Abe et al. 1990, Shibuya et al. 1991). In addition to its immunomodulatory action, bestatin has been studied extensively for many years because of its anti-tumor effect (Tsukagoshi 1987, Ota and Uzuka 1992, Ichnose et al. 2003, Ichimura et al. 2006). A lot of clinical trials confirmed that bestatin in combination with chemotherapy prolongs both the remission period and survival time of patients with leukemia (Ota and Uzuka 1992, Urabe et al. 1993, Hirayama et al. 2003). There are also interesting and promising evidences that bestatin has an adjuvant effect on a DNA AIDS vaccine (Sasaki et al. 1998) and may hinder HIV infection (Pulido-Cejudo et al. 1997).

It is believed that the action of bestatin is connected with its ability to inhibit the enzyme – aminopeptidase, especially aminopeptidase N (APN) (Bauvois et al. 2006).

Aminopeptidase N is a metalloprotease belonging to the M1 family of the MA clan of peptidases (Luan and Xu 2007). It is identical with 150-kDa cell surface glycoprotein – CD13 (Look et al. 1989). There are two forms of APN – soluble aminopeptidase N, found in plasma/serum (Favaloro et al. 1993) and urine (Jung et al. 1984) and membrane-bound APN (Luan and Xu 2007). The enzyme is widely distributed in hematopoietic cells of myeloid origin as well as in epithelial-, endothelial- and fibroblast- type cells. A high expression level of APN/CD13 has been reported in various inflammatory, neoplasic and CNS diseases. For this reason, it can be regarded as a useful clinical marker. Besides, pharmacological inhibition of APN/CD13 may give good therapeutic results in the treatment of these diseases (Bauvois et al. 2006).

The purpose of the present study was to determine the effects of bestatin, an inhibitor of APN/CD13, on the surface marker expression of the thymus, spleen and mesenteric lymph node cells with respect to the dosage and schedule of treatment in mice.

Materials and Methods

Animals

The experiments were carried out on female Balb/c mice, each weighing 18-20 g (7-8 weeks of age). The mice were kept under conventional conditions and had *ad libitum* access to water and granulated food. They were obtained from a Breeding Center of Laboratory Animals of the Instituite of Occupational Medicine, Łódź, Poland. The principles of laboratory animal care (NIH publication No. 86-23, revised 1985) as well as the specific national laws on the protection of animals were followed. The study protocol was approved by the II Local Ethics Commission in Wrocław, Poland (No.14/2007)

Treatment

Bestatin (Sigma-Aldrich Chemie GmbH, Riedstr. 2,D-89555 Steiheim, Germany, in powder form) was dissolved in phosphate-buffered saline (PBS, Institute of Immunology and Experimental Therapy, Wrocław, Poland). The agent was administered intraperitoneally (*i.p.*) once, five or ten times at 24 h intervals at three different doses of 10, 1 and 0.1 mg/kg. The trials on the control mice were conducted in parallel. The mice in the control group received phosphate-buffered saline solution (PBS) alone. The volume of each dose of bestatin or PBS was 0.2 ml per animal. Each control and experimental group consisted of seven mice.

Measurements

The measurements included: (i) the total number of thymocytes, splenocytes and lymphocytes of mesenteric lymph nodes; (ii) the weight ratio of thymus, spleen and mesenteric lymph nodes calculated according to the following formula: weight of organ (g)/body weight of mouse (g) × 100; (iii) the percentage and count of lymphocyte subpopulations in lymphatic organs.

The total number of thymocytes, splenocytes, lymphocytes of mesenteric lymph nodes, the weight ratio of the thymus, spleen and mesenteric lymph nodes, the percentage and count of CD subsets ($CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+$, $CD8^+$ in the thymus, $CD19^+$, $CD3^+$, $CD4^+$ and $CD8^+$ in the spleen and mesenteric lymph nodes) were determined 24 hours after the last bestatin administration.

Assays of thymocyte, splenocyte and lymphocyte of mesenteric lymph node subsets

The mice were anaesthetized with halothane (Narcotan, Zentiva, Prague, Czech Republic) 24 hours after the final dose of bestatin administration. Thymuses, spleens and mesenteric lymph nodes were removed and placed in disposable Petri dishes containing a sterile, ice-cold PBS. The suspended cells were released from the lymphatic organs by gentle passage through a nylon mesh and then centrifuged (2250 x g, 15 min, 4°C) on a layer of Ficoll 400 (Pharmacia, Fine Chemicals AB, Uppsala, Sweden)/ Urografin 76% (diatrizoate sodium and meglumine diatrizoate, Bayer Schering Pharma, Poland) in a 1 : 3 ratio at a density of 1.076. After centrifugation, the cells were collected from the interphase and washed twice (375 x g, 8 min, 4°C) with a sterile, ice-cold PBS supplemented with 1% bovine serum albumin (BSA, Sigma). After the second wash, the cells were suspended in PBS with 1% BSA at 1×10^7 cells/ml. The viability of each cell suspension was 90-98% according to a trypan blue dye-exclusion assay. The cells were resuspended in 100 µl of PBS containing 1% BSA. The thymocytes, splenocytes and lymphocytes of the mesenteric lymph nodes were stained with Rat Anti-Mouse CD4: FITC / CD8: RPE dual color reagent (Serotec, Kidlington, UK) at the dilution recommended by the manufacturer. The splenocytes and lymphocytes of the mesenteric lymph nodes were also stained with Rat Anti-Mouse CD19:FITC / CD3:RPE dual color reagent (Serotec, Kidlington, UK) at a dilution recommended by the producer.

The cells were incubated at 4°C for 30 min. and washed (375 x g, 8 min, 4°C) three times with an ice-cold PBS. The fluorescence was analyzed using a flow cytometer (FACS Calibur; Becton Dickinson Biosciences, San Jose, CA, USA). Data acquisition and analysis were done using a CellQuest 3.1f software. A two-color analysis was performed: fluorescence 1 (FL1) – FITC: emission peak 525 nm, fluorescence 2 (FL2) – PE: emission peak 575 nm. Instrument settings used in this study were as follows: FL1: log, 584V, FL2: log, 595V, the fluorescence compensation: FL1: -1.7 % FL2; FL2: -22.3 % FL1. A total of 10 000 events were collected.

Determination of the total number of thymocytes, splenocytes and mesenteric lymph node lymphocytes

The lymphatic organs after removing from anaesthetized with halothane mice were passed through a nylon mesh into 1 ml of a sterile, ice-cold PBS. Next, the suspended cells were diluted ten times in PBS. The number of mononucleated cells from central and peripheral lymphatic organs was counted in a Thoma hemocytometer using Turk solution.

Statistical analysis

The data obtained in the study were analyzed statistically using a t-Student test. The differences were considered significant at $p < 0.05$.

Results

The effects of bestatin on the total number of lymphocytes in the thymus, spleen and mesenteric lymph nodes and the weight ratio of lymphatic organs in mice

As can be seen in Table 1A, single bestatin doses of 10, 1 or 0.1 mg/kg neither changed the total number of lymphocytes in the thymus, spleen and mesenteric lymph nodes nor the weight ratio of these lymphatic organs in mice. However, the total number of thymocytes in mice markedly increased 24 hours after a repeated exposure to bestatin. The stimulating effect of the agent was independent of the dosage and the number of subsequent doses applied. Moreover, ten times administration of bestatin at doses of 10, 1, and 0.1 mg/kg also significantly increased the total number of lymphocytes in the spleen and mesenteric lymph nodes, but no changes were observed in the total number of lymphocytes in the lymphatic organs after five subsequent bestatin doses (Tables 1B and 1C). Five exposures to bestatin at a dose of 10 mg/kg increased the weight ratio of the thymus. The weight ratio of the spleen also significantly increased as early as 24 hours following the exposure to five bestatin doses of 1 and 0.1 mg/kg. However, the weight ratio of the mesenteric lymph nodes also increased 24 hours after ten bestatin doses.

The effects of bestatin on the percentage and absolute number of lymphocyte subpopulations in the thymus, spleen and mesenteric lymph nodes in mice

It has been found that bestatin is able to change the percentage and absolute number of T cell subsets in the thymus and T and B lymphocytes in the spleen and mesenteric lymph nodes. The effect of the drug is dependent on the dosage and the number of consecutive doses applied.

As can be seen in Table 2, bestatin administered at a single dose of 10 mg/kg increased the percentage and absolute count of immature $CD4^-CD8^-$ thymic

Table 1. The effects of bestatin with respect to dosage and schedule of treatment on the total number of thymocytes, splenocytes and mesenteric lymph nodes cells and weight ratio of thymus, spleen and mesenteric lymph nodes. The mean values (n=7) and standard deviations are presented.

A. Administration of bestatin in a single dose of 10, 1 or 0.1 mg/kg

Index	Control	Bestatin		
		1 x 10 mg/kg	1 x 1 mg/kg	1 x 0.1 mg/kg
The total number of thymocytes (x 10^7)	31.9 ± 3.8	26.5 ± 4.0	29.9 ± 6.9	33.0 ± 7.3
Weight ratio of thymus	0.206 ± 0.035	0.199 ± 0.03	0.185 ± 0.048	0.228 ± 0.062
The total number of splenocytes (x10^7)	56.7 ± 6.9	54.2 ± 11.0	57.8 ± 6.3	60.1 ± 5.5
Weight ratio of spleen	0.548 ± 0.091	0.625 ± 0.135	0.62 ± 0.11	0.64 ± 0.098
The total number of mesenteric lymph node cells (x10^7)	35.6 ± 6.5	35.2 ± 6.2	37.2 ± 4.4	34.7 ± 5.9
Weight ratio of mesenteric lymph nodes	0.448 ± 0.131	0.374 ± 0.035	0.466 ± 0.067	0.4 ± 0.1

B. Administration of bestatin five times at 24 h intervals at doses of 10, 1 or 0.1 mg/kg

Index	Control	Bestatin		
		5 x 10 mg/kg	5 x 1 mg/kg	5 x 0.1 mg/kg
The total number of thymocytes (x 10^7)	23.3 ± 4.6	29.1 ± 6.5	32.4 ± 2.6*	32.4 ± 3.1*
Weight ratio of thymus	0.15 ± 0.02	0,202 ± 0.044*	0.185 ± 0.036	0.192 ± 0.055
The total number of splenocytes (x10^7)	52.2 ± 5.5	52.0 ± 8.3	55.9 ± 5.2	51.6 ± 6.4
Weight ratio of spleen	0.437 ± 0.064	0.496 ± 0.052	0.572 ± 0.116*	0.552 ± 0.101*
The total number of mesenteric lymph node cells (x10^7)	34.7 ± 4.2	37.5 ± 5.4	37.6 ± 5.9	36.8 ± 4.1
Weight ratio of mesenteric lymph nodes	0.425 ± 0.114	0.404 ± 0.067	0.367 ± 0.084	0.416 ± 0.094

C. Administration of bestatin ten times at 24 h intervals at doses of 10, 1 or 0.1 mg/kg

Index	Control	Bestatin		
		10 x 10 mg/kg	10 x 1 mg/kg	10 x 0.1 mg/kg
The total number of thymocytes (x 10^7)	28.0 ± 4.6	37.3 ± 5.1*	38.5 ± 5.2*	38.8 ± 4.5*
Weight ratio of thymus	0.225 ± 0.028	0.2 ± 0.04	0.204 ± 0.033	0.249 ± 0.055
The total number of splenocytes (x10^7)	51.3 ± 3.8	69.8 ± 5.7*	64.9 ± 9.5*	60.5 ± 3.0*
Weight ratio of spleen	0.627 ± 0.074	0.7 ± 0.05	0.62 ± 0.11	0.623 ± 0.03
The total number of mesenteric lymph node cells (x10^7)	34.0 ± 3.8	39.1 ± 2.2*	38.3 ± 3.1*	37.0 ± 2.4
Weight ratio of mesenteric lymph nodes	0.339 ± 0.04	0.486 ± 0.131*	0.462 ± 0.169	0.446 ± 0.11**

$p < 0.05$ as compared to the control group

cells (double-negative cells) and decreased the percentage and absolute count of immature $CD4^+CD8^+$ thymocytes (double-positive cells). Bestatin injected once changed neither the percentage nor the absolute count of single-positive, mature $CD4^+$ and $CD8^+$ thymic cells, irrespectively of the dose applied. Five and ten exposures to the bestatin doses under investigation significantly increased the absolute count of both the immature double-positive ($CD4^+CD8^+$) and double-negative ($CD4^-CD8^-$) thymic cells, but the influence of bestatin on the percentage of these subsets of thymocytes was insignificant (Tables 3, 4 and Figure 1). However, a decrease in the percentage, but no change of the absolute number of mature, single-positive $CD4^+$ thymocytes was observed.

Table 2. Percentage and absolute number of thymocytes, splenocytes and lymphocytes from mesenteric lymph node subpopulations in mice treated with bestatin administered once. The mean values (n=7) and standard deviations are presented.

Index		Control	Bestatin		
			1 x 10 mg/kg	1 x 1 mg/kg	1 x 0.1 mg/kg
Thymocytes					
$CD4^-CD8^-$	(%)	5.0 ± 1.4	8.7 ± 1.6*	6.5 ± 1.7	6.7 ± 0.6
	($x10^7$)	1.6 ± 0.4	2.3 ± 0.6*	1.9 ± 0.5	2.2 ± 0.6*
$CD4^+CD8^+$	(%)	66.1 ± 3.9	60.5 ± 3.4*	63.5 ± 4.1	65.5 ± 5.1
	($x10^7$)	21.2 ± 3.2	16.0 ± 2.4*	19.1 ± 5.3	21.5 ± 4.5
$CD4^+$	(%)	23.5 ± 2.5	25.1 ± 2.2	24.3 ± 1.5	22.1 ± 3.3
	($x10^7$)	7.5 ± 1.0	6.6 ± 1.2	7.2 ± 1.5	7.4 ± 2.2
$CD8^+$	(%)	5.3 ± 0.3	5.7 ± 0.6	5.7 ± 1.1	5.7 ± 1.4
	($x10^7$)	1.7 ± 0,2	1.5 ± 0.3	1.7 ± 0,3	1.9 ± 0.7
Splenocytes					
$CD3^+$	(%)	30.4 ± 7.8	19.5 ± 4.3*	24.6 ± 3.6	21.4 ± 1.3*
	($x10^7$)	17.2 ± 5.0	10.6 ± 3.4*	14.2 ± 2.2	12.8 ± 1.2
$CD4^+$	(%)	23.9 ± 5,6	17.8 ± 3.5*	20.8 ± 2.8	21.7 ± 4.3
	($x10^7$)	13.6 ± 4.2	9.6 ± 2.7	11.9 ± 1.1	13.0 ± 2,9
$CD8^+$	(%)	5.3 ± 1.7	3.8 ± 1.1	3.9 ± 0.6	3.6 ± 1.1*
	($x10^7$)	3.0 ± 1.1	2.0 ± 0.8	2.3 ± 0.2	2.1 ± 0,7
$CD19^+$	(%)	53.6 ± 10.2	68.7 ± 6.1*	58.9 ± 4.7	61.7 ± 7.6
	($x10^7$)	30.5 ± 7.3	37.2 ± 8.1	34.0 ± 4.6	36.8 ± 6.4
Mesenteric lymph node cells %					
$CD3^+$	(%)	54.4 ± 6.6	58.6 ± 3.8	45.8 ± 4.8*	45.9 ± 4.7*
	($x10^7$)	19,3 ± 4.3	20.6 ± 3.7	17.1 ± 3.3	15.8 ± 3.0
$CD4^+$	(%)	47.9 ± 6.0	50.1 ± 3.6	39.7 ± 3.1*	39.3 ± 3.7*
	($x10^7$)	17.0 ± 3.8	17.6 ± 3.3	14.8 ± 2.1	13.7 ± 2.9
$CD8^+$	(%)	9.0 ± 2.2	9.8 ± 2.5	5.6 ± 0.9*	7.2 ± 2.6
	($x10^7$)	3.2 ± 1.0	3.4 ± 1.0	2.1 ± 0.4*	2.5 ± 0.8
$CD19^+$	(%)	39.0 ± 8.6	35.8 ± 4.0	48.3 ± 4.7*	48.9 ± 7.5*
	($x10^7$)	13.8 ± 4.1	12.6 ± 2.7	17.8 ± 1.7*	17.0 ± 3.8

* $p < 0.05$ as compared to the control group

At the same time, some changes in the percentage and the absolute number of T and B cells in the spleen and mesenteric lymph nodes were found (Tables 2-4 and Figures 2-5). A single administration of bestatin at a dose of 10 mg/kg reduced the percentage of $CD3^+$ (Pan-T-cells) splenocytes, which corresponded with a decreased percentage of $CD4^+$ and $CD8^+$ and an increased percentage of $CD19^+$ cells (B lymphocytes) in the spleen. Similar effects of single bestatin doses of 1 or 0.1 mg/kg on the percentage of $CD3^+$, $CD4^+$, $CD8^+$ and $CD19^+$ lymphocytes in mesenteric lymph nodes were observed (Table 2). However, no changes in the absolute count of T and B lymphocytes in the spleen and mesenteric lymph nodes were observed (except a decrease in the absolute count of $CD3^+$ splenocytes after a single bestatin dose of 10 mg/kg and a decrease in the absolute count of $CD8^+$ cells and an increase in the absolute count of $CD19^+$ lymphocytes in mesenteric lymph nodes after bestatin administration at a single dose of 1 mg/kg),

Table 3. Percentage and absolute number of thymocytes, splenocytes and lymphocytes from mesenteric lymph node subpopulations in mice treated with bestatin administered five times. The mean values (n=7) and standard deviations are presented.

Index		Control	Bestatin		
			5 x 10 mg/kg	5 x 1 mg/kg	5 x 0.1 mg/kg
Thymocytes					
$CD4^-CD8^-$	(%)	2.3 ± 0.7	2.2 ± 0.4	2.4 ± 0.3	2.5 ± 0.6
	($x10^7$)	0.5 ± 0.1	0.6 ± 0.1	0.8 ± 0.1*	0.8 ± 0.2*
$CD4^+CD8^+$	(%)	80.4 ± 1.6	82.0 ± 1.6	82.8 ± 2.4	82.1 ± 2.9
	($x10^7$)	18.7 ± 3.7	23.9 ± 5.6	26.8 ± 2.4*	26.6 ± 2.8*
$CD4^+$	(%)	14.6 ± 1.1	13.1 ± 1.1*	12.5 ± 1.9*	12.8 ± 2.1
	($x10^7$)	3.4 ± 0.8	3.8 ± 0.7	4.0 ± 0.6	4.2 ± 0.8
$CD8^+$	(%)	2.7 ± 0.2	2.6 ± 0.2	2.3 ± 0.3*	2.5 ± 0.6
	($x10^7$)	0.6 ± 0.1	0.8 ± 0.2	0.7 ± 0.1	0.8 ± 0.2
Splenocytes					
$CD3^+$	(%)	38.0 ± 6.9	32.1 ± 6.0	27.8 ± 5.1*	22.7 ± 7.2*
	($x10^7$)	20.0 ± 4.9	18.3 ± 4.4	14.6 ± 2.5*	10.5 ± 3.6*
$CD4^+$	(%)	28.0 ± 4.4	23.3 ± 4.2	22.7 ± 4.0*	17.2 ± 4.2*
	($x10^7$)	14.7 ± 3.3	13.3 ± 2.9	12.5 ± 1.6	9.1 ± 3.2*
$CD8^+$	(%)	7.1 ± 1.7	5.4 ± 1.7	3.8 ± 0.9*	2.2 ± 0.4*
	($x10^7$)	3.7 ± 1.1	2.9 ± 1.2	2.1 ± 0.3*	1.1 ± 0.2*
$CD19^+$	(%)	53.0 ± 8.0	58.7 ± 5.7	59.9 ± 7.2	71.2 ± 7.7*
	($x10^7$)	27.5 ± 4.0	30.2 ± 3.5	33.7 ± 6.5	36.3 ± 2.3*
Mesenteric lymph node cells					
$CD3^+$	(%)	58.1 ± 6.8	45.1 ± 7.5	34.9 ± 4.0*	37.8 ± 4.9*
	($x10^7$)	20.0 ± 2.4	17.5 ± 2.8	13.0 ± 1.4*	14.0 ± 2.9*
CD4+	(%)	46.1 ± 2.5	41.5 ± 7.5	31.8 ± 4.0*	33.8 ± 4.4*
	($x10^7$)	15.9 ± 1.6	15.3 ± 1.8	11.9 ± 2.1*	12.3 ± 1.3*
CD8+	(%)	10.8 ± 4.8	4.6 ± 0.4*	3.7 ± 1.0*	4.0 ± 0.9*
	($x10^7$)	3.7 ± 1.7	1.8 ± 0.2*	1.4 ± 0.4*	1.4 ± 0.3*
CD19+	(%)	37.6 ± 6.9	50.4 ± 7.9*	59.6 ± 5.1*	45.8 ± 11.1
	($x10^7$)	13.2 ± 3.7	19.7 ± 4.1*	22.6 ± 5.1*	16.8 ± 4.4*

$p < 0.05$ as compared to the control group

since a single administration of this agent did not have a significant impact on the total number of lymphocytes in the spleen and mesenteric lymph nodes. As can be seen in Table 3, five exposures to bestatin (1 or 0.1 mg/kg) at 24 hour intervals decreased the percentage and absolute count of $CD3^+$ splenocytes and mesenteric lymph node cells which corresponded with the decreases in the percentage and absolute count of $CD4^+$ and $CD8^+$ cells. Exposure to ten doses of bestatin at 24 hour intervals was less activating than five injections. Administration of ten bestatin doses of 1 or 0.1 mg/kg significantly increased the percentage and absolute count of $CD8^+$ lymphocytes (suppressive and cytotoxic T cells) in the mesenteric lymph nodes (Table 4). Multiple administration of bestatin at all doses under investigation augmented the percentage and absolute count of $CD19^+$ (B lymphocytes) in the spleen and mesenteric lymph nodes. The strongest stimulating effect on B lymphocytes of the spleen was observed after five bestatin injections at 0.1 mg/kg doses. This effect was also observed on B lymphocytes of mesenteric lymph nodes after five bestatin injections at higher doses (10 or 1 mg/kg) (Table 3).

Table 4. Percentage and absolute number of thymocytes, splenocytes and lymphocytes from mesenteric lymph node subpopulations in mice treated with bestatin administered ten times. The mean values (n=7) and standard deviations are presented.

Index		Control	Bestatin		
			10 x 10 mg/kg	10 x 1 mg/kg	10 x 0.1 mg/kg
Thymocytes					
$CD4^-CD8^-$	(%)	2.5 ± 0.7	2.2 ± 0.9	4.7 ± 2.4	2.4 ± 0.3
	(x 10^7)	0.7 ± 0.2	0.8 ± 0.3	1.8 ± 1.0*	0.9 ± 0.1
$CD4^+CD8^+$	(%)	76.5 ± 1.8	78.3 ± 2.6	77.0 ± 2.0	79.4 ± 2.2*
	(x 10^7)	21.4 ± 3.5	29.3 ± 4.8*	29.6 ± 3.8*	30.9 ± 4.2*
$CD4^+$	(%)	18.2 ± 1.2	16.2 ± 0.8*	15.4 ± 1.3*	15.2 ± 1.8*
	(x 10^7)	5.1 ± 0.9	6.0 ± 0.7	5.9 ± 1.1	5.8 ± 0.3
$CD8^+$	(%)	2.8 ± 0.7	2.8 ± 0.5	2.9 ± 0.4	3.0 ± 0.5
	(x 10^7)	0.8 ± 0.2	1.0 ± 0.1*	1.1 ± 0.3*	1.1 ± 0.2*
Splenocytes					
$CD3^+$	(%)	42.4 ± 7.2	40.0 ± 6.0	38.3 ± 6.4	36.2 ± 6.6
	(x 10^7)	23.1 ± 2.3	26.5 ± 3.0	24.9 ± 6.0	21.8 ± 3.7
$CD4^+$	(%)	33.9 ± 5.6	29.6 ± 4.1	28.7 ± 3.2	28.3 ± 4.5
	(x 10^7)	17.4 ± 3.5	20.7 ± 3.3	18.8 ± 4.2	17.1 ± 2.9
$CD8^+$	(%)	7.1 ± 1.6	6.3 ± 1.4	4.8 ± 1.2*	4.6 ± 0.9*
	(x 10^7)	3.7 ± 0.9	4.4 ± 1.2	3.1 ± 0.8	2.8 ± 0.6*
$CD19^+$	(%)	49.7 ± 7.4	53.5 ± 5.9	55.5 ± 6.6	56.0 ± 7.2
	(x 10^7)	25.5 ± 4.0	37.4 ± 5.6*	35.9 ± 6.5*	34.0 ± 5.5*
Mesenteric lymph node cells					
$CD3^+$	(%)	57.3 ± 7.2	48.4 ± 5.1*	62.5 ± 5.1	58.5 ± 4.6
	(x 10^7)	19.3 ± 1.9	18.9 ± 2.2	24.0 ± 2.9*	21.7 ± 2.5
$CD4^+$	(%)	46.8 ± 7.7	40.4 ± 4.3	44.3 ± 3.2	43.1 ± 4.4
	(x 10^7)	15.8 ± 2.8	15.8 ± 1.8	17.0 ± 1.6	16.0 ± 2.4
$CD8^+$	(%)	6.7 ± 1.1	4.7 ± 1.5*	13.1 ± 1.2*	13.8 ± 1.0*
	(x 10^7)	2.3 ± 0.4	1.8 ± 0.6	5.0 ± 0.6*	5.1 ± 0.2*
$CD19^+$	(%)	39.3 ± 7.5	48.7 ± 5.1*	32.5 ± 4.3	36.4 ± 4.1
	(x 10^7)	13.5 ± 3.7	19.0 ± 2.3*	12.4 ± 1.8	13.5 ± 1.6*

$p < 0.05$ as compared to the control group

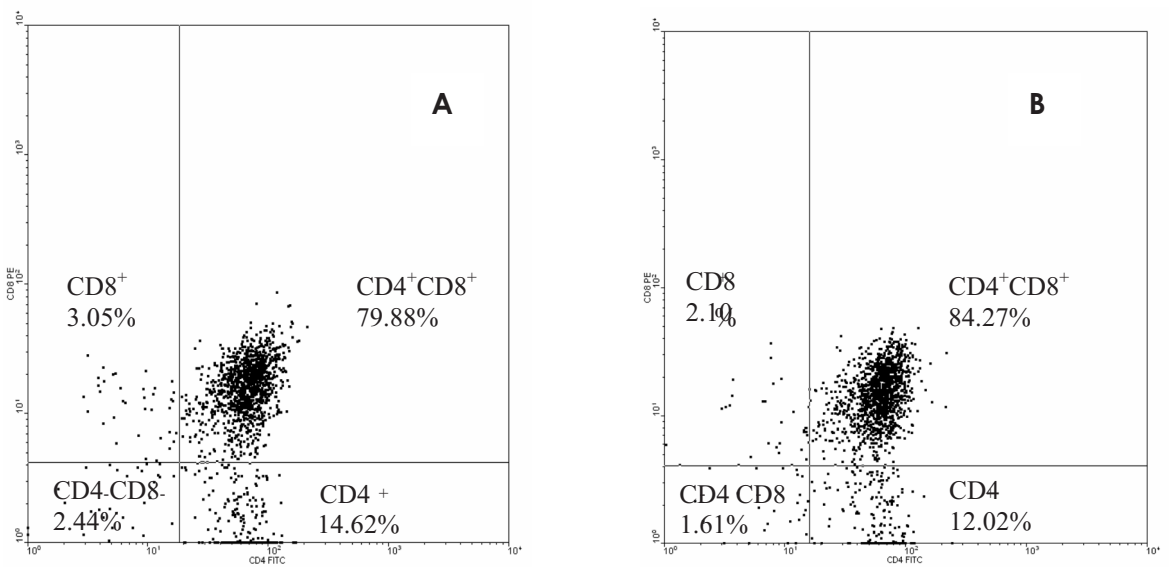

Fig. 1. Examples of dot plots of thymocyte subpopulations A) control group B) bestatin administered five times at a dose of 1 mg/kg (after 24 h).

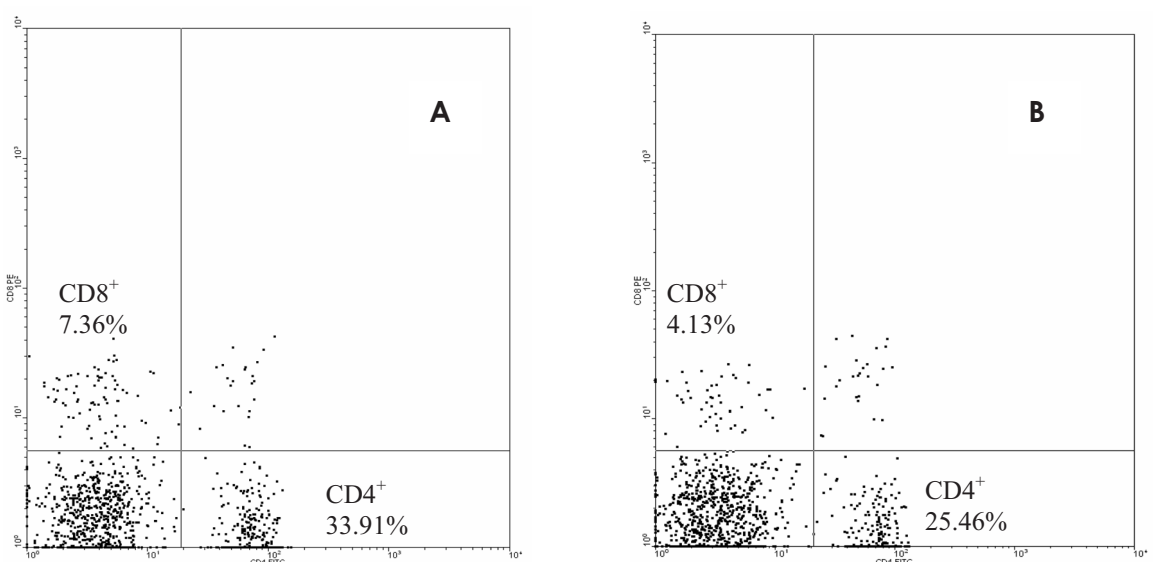

Fig. 2. Examples of dot plots of splenocyte subpopulations A) control group B) bestatin administered five times at a dose of 1 mg/kg (after 24 h).

Discussion

The results obtained in the present study conducted on mice show that bestatin, a potent aminopeptidase inhibitor, is able to change the total number of lymphocytes in the thymus, spleen and mesenteric lymph nodes and the percentage and the absolute number of T-cell subsets in the thymus and T and B lymphocytes in the spleen and mesenteric lymph nodes. The results show a relationship between the modulating effect induced by bestatin and the dose of the drug as well as the number of doses applied. The results obtained in the present study show that multiple exposures to bestatin significantly increased the absolute count of both immature double-positive ($CD4^+CD8^+$) and double-negative ($CD4^-CD8^-$) thymic cells and decreased the percentage and absolute count of $CD3^+$ splenocytes and mesenteric lymph node cells which corresponded with the decreases in the percentage and absolute count of $CD4^+$ and $CD8^+$ cells. Exposure to ten doses of bestatin at 24 hour intervals was less effective than five injections. The influence of bestatin on the count and function of T lymphocytes was also found by other authors.

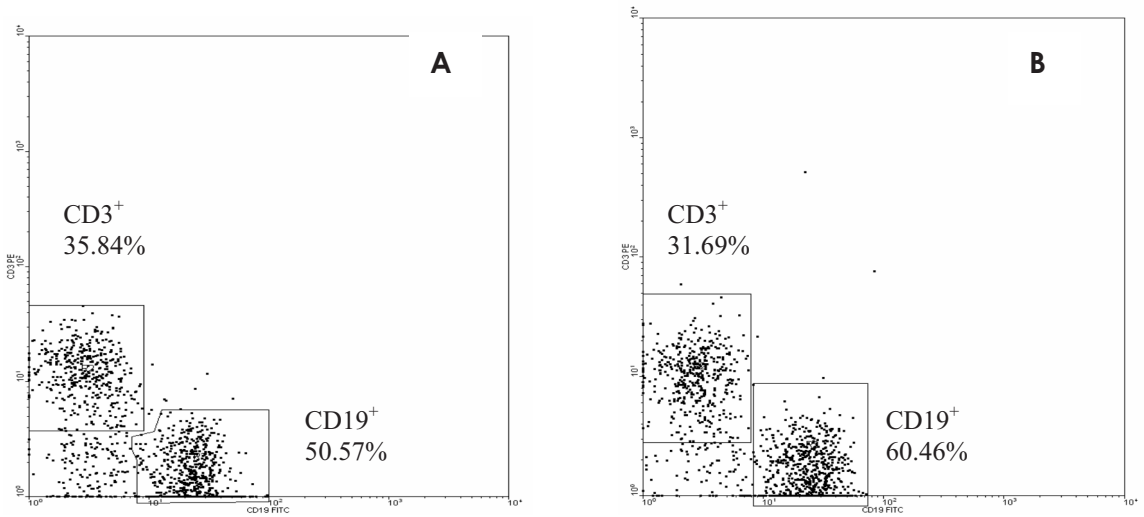

Fig. 3. Examples of dot plots of splenocyte subpopulations A) control group B) bestatin administered five times at a dose of 1 mg/kg (after 24 h).

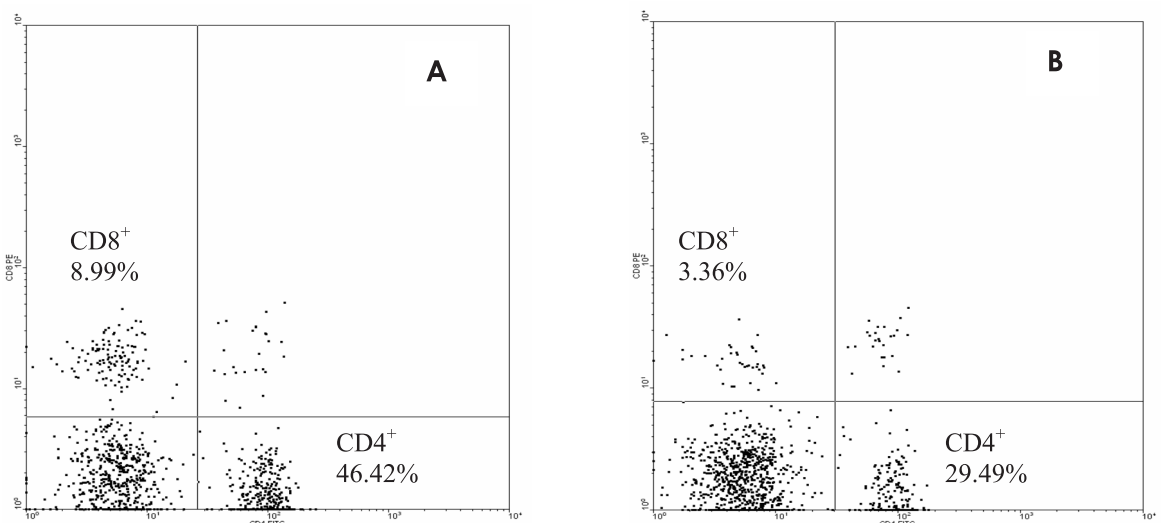

Fig. 4. Examples of dot plots of mesenteric lymph nodes lymphocyte subpopulations A) control group B) bestatin administered five times at a dose of 1 mg/kg (after 24 h).

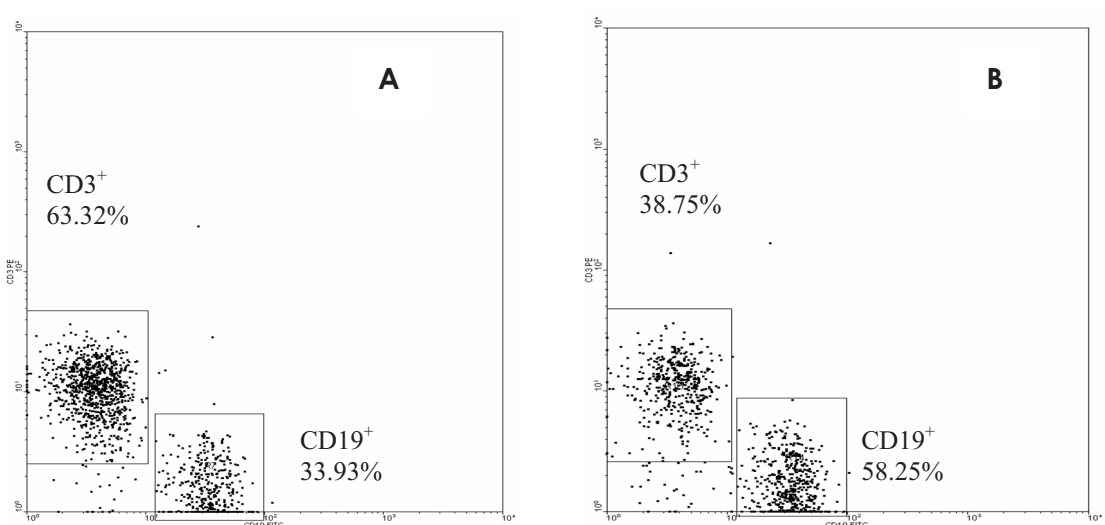

Fig. 5. Examples of dot plots of mesenteric lymph nodes lymphocyte subpopulations A) control group B) bestatin administered five times at a dose of 1 mg/kg (after 24 h).

Yamagishi et al. (1991) reported changes in cellular immunity in gastrointestinal patients after bestatin treatment. They found that oral administration of bestatin at a dose of 30 mg for 1-2 months increased the absolute count of helper and cytotoxic T cells, IL-2 receptor, PHA blastogenesis, PPD (*purified protein derivative from tuberculosis*) skin reaction, but decreased the absolute count of suppressor T cells. Dunlap et al. (1984) found that bestatin at a concentration of 25 μg/ml in mixed lymphocyte cultures of murine spleen cells increased the incorporation of [^{3}H] thymidine into these cells. However, bestatin used at a concentration of 5 μg/ml inhibited the response of thymocytes and splenocytes to concanavalin A (Con A) stimulation in BALB/c mice. This result led the authors to the hypothesis that bestatin exerts both a stimulatory and an inhibitory influence on the immune response and that the final effect depends on such factors as particular assay, culture conditions and species. This observation could also explain the results obtained in our experiments. On the other hand, it is assumed that bestatin exerts a stimulating effect on T cells proliferation through activation of macrophages. However, it is not fully elucidated whether bestatin first activates the production of lymphokines by T cells, thus stimulating the macrophages – which consequently cause T cell proliferation, or bestatin affects macrophages directly and these cells stimulate T lymphocyte proliferation (Ishizuka et al. 1980b).

It has been found that bestatin enhances the release of IL-2 by murine and rat as well as human lymphocytes. This cytokine is considered to be a T cell growth factor (Nelson and Willerford 1998). Hence, it seems likely that the stimulating effect of bestatin on the percentage and absolute count of T lymphocytes is related to the synthesis and release of IL-2. On the other hand, Wakabayashi et al. (1991) found that bestatin had no influence on the overall number of precursor T cells, but it changed the proportion between particular subpopulations. The drug increased $CD4^+$ and $CD4^+CD8^+$ cells and decreased $CD8^+$ cells. These results indicate that bestatin induces the differentiation of precursor T cells into $CD4^+$ lymphocytes. The results of our studies conducted on cyclophosphamide-suppressed mice indicate that the influence of bestatin on T lymphocyte subsets in peripheral lymphatic organs depends on the dose of the drug and the number of doses applied as well as the immunologic status of the host. Five or ten times exposure to bestatin (at doses of 1 or 0.1 mg/kg) after a single dose of cyclophosphamide (350 mg/kg) induced an immunocorrecting action resulting in an increased percentage and absolute count of $CD3^+$, $CD4^+$ and $CD8^+$ cells in the spleen and mesenteric lymph nodes of mice. The strongest correcting effect was observed with ten exposures to bestatin at a dose of 0.1 mg/kg after cyclophosphamide administration (Lis, unpublished doctoral dissertation 2009).

The results of the present study also indicate that bestatin increased the percentage and absolute count of B lymphocytes ($CD19^+$ cells) in the spleen and mesenteric lymph nodes, irrespective of the dosage and the number of doses applied.

Otsuka et al. (1988) reported that bestatin *in vitro* at concentrations of 1 and 0.1 μg/ml significantly increased the number of B cell colonies. The effect was observed only in the presence of irradiated T cells as feeders, but not without these cells present in the culture. Besides, soluble factor production induced by PHA (*phytohemagglutinin*)-stimulated T cells increased in the presence of bestatin. It can, therefore, be concluded that the stimulating effect of bestatin on B cell colony formation is mediated by T lymphocytes. However, Morikawa et al. (1989) reported that bestatin at concentration ranges of 4-225 μg/ml suppressed the proliferation and differentiation of human B cells *in vitro*. This effect was not achieved by the addition of BCGF (*B cell growth factor*) or IL-2 and has never been found in human B lymphoblastoid cell lines.

The differences in the above findings could result from the differences in the purity of the target cells, different route of administration or assay system. Besides, the presence of other cells, such as natural killer cells, monocytes, or T cells, on which bestatin exerts a stimulating effect, may influence the action of the drug on B lymphocytes.

In conclusion, it can be stated that the anti-aminopeptidase action of bestatin on the total number of lymphocytes in the thymus, spleen and lymph nodes and T and B lymphocyte subsets in the lymphatic organs of mice depends on the dosage and the number of doses applied. Multiple administration of bestatin significantly increased the total number of lymphocytes in the spleen and mesenteric lymph nodes. Bestatin decreased the percentage and absolute count of $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes, but increased the percentage and absolute count of $CD19^+$ lymphocytes 24 hours after the final administration.

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